Ascorbic acid inhibition of *Candida albicans* Hsp90-mediated morphogenesis occurs via the transcriptional regulator Upc2.

Running title: Ascorbic acid affects elongation upon Hsp90 compromise

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Abstract

Morphogenetic transitions of the opportunistic fungal pathogen *Candida albicans* are influenced by temperature changes, with induction of filamentation upon a shift from 30 to 37°C. Hsp90 was identified as a major repressor of an elongated cell shape morphology at low temperatures, as treatment with specific inhibitors of Hsp90 results in elongated growth forms at 30°C. Elongated growth resulting from a compromised Hsp90 is considered neither hyphal nor pseudohyphal growth. It has already been reported that ascorbic acid (Vitamin C) interferes with the yeast-to-hyphae transition in *C. albicans*. In the present study, we show that ascorbic acid also antagonizes the morphogenetic change caused by hampered Hsp90 function. Further analysis revealed that Upc2, a transcriptional regulator of genes involved in ergosterol biosynthesis, and Erg11, the target of azole antifungals whose expression is in turn regulated by Upc2, are required for this antagonism. Ergosterol levels correlate with elongated growth, being reduced in cells treated with the Hsp90 inhibitor Geldanamycin (GdA), and restored by co-treatment with ascorbic acid.

In addition, we illustrate that Upc2 appears to be required for ascorbic acid-mediated inhibition of the antifungal activity of fluconazole. These results identify Upc2 as a major regulator of ascorbic acid-induced effects in *C. albicans* and suggest an association between ergosterol content and elongated growth upon Hsp90 compromise.

Introduction

Candida albicans belongs to the few fungal species that are frequently encountered as clinical pathogens (1). *C. albicans* is a frequent commensal of healthy individuals but when the host immune system is weakened, it can become a deadly pathogen (2). Its pathogenic nature relies on a multitude of factors, including the ability to grow at 37 °C, to produce adhesins and hydrolases and to change between different morphological forms, including yeast cells, hyphae, pseudohyphae, chlamydospores, opaque cells and the recently described GUT morphology (3-5). It is generally assumed that the yeast form is crucial for fungal dissemination via the bloodstream throughout the body (6), while hyphae on the other hand are required for the production of extracellular enzymes and invasive growth (7).

The yeast-to-hyphae transition of *C. albicans* can be triggered by environmental cues such as pH, serum, elevated temperature and CO_2 (4, 8). In addition several small molecules, such as autoregulatory molecules, cell cycle inhibitors and histone deacetylase inhibitors, are also capable of modulating morphogenetic responses (9, 10). Most environmental triggers and small molecules function through activation of the cAMP-PKA or MAPK pathways via their downstream transcription factors Efg1 and Cph1, respectively (4, 11, 12). Over the past few years, several reports appeared that showed that Geldanamycin (GdA), a benzoquinone ansamycin antibiotic is another small molecule that strongly affects the cell shape of *C. albicans* at 30 °C as its addition results in elongated cells. GdA inhibits the function of Hsp90 by binding to its ADP/ATP binding pocket (10, 13) indicating that Hsp90 prevents an elongated cell shape of *C. albicans* at lower temperatures. This morphogenetic process also involves the Ras1-

67 cAMP-PKA signalling pathway but it seems to do this independent of the downstream
68 transcription factor Efg1, suggesting the involvement of other transcriptional regulators
69 (13). Screening of a transcription factor (TF) deletion collection for mutants impaired in
70 the Hsp90-mediated elongated cell shape phenotype, resulted in the identification of
71 Hms1 (14). Two upstream regulators of this TF, the cyclin-dependent kinase Pho85 and
72 the cyclin Pcl1 are also required for elongated cell shape (14). As Hms1 is not a
73 component of the cAMP-PKA pathway, the link between Hsp90-dependent elongated
74 cell shape and the Ras1-PKA signalling pathway remained to be identified. Our aim in
75 this work was to identify transcription factors that upon overexpression affect the Hsp90-
76 depended elongated cell shape. For this we screened a TF overexpression library and we
77 found that overexpression of Upc2 prevents the elongated growth phenotype when GdA
78 is added. The Zn_2Cys_6 transcriptional regulator Upc2 is important for the regulation of
79 the ergosterol biosynthetic pathway in response to commonly used antifungals (15). This
80 transcription factor can act as a repressor or an activator depending on its target and on
81 the initiating conditions, and exerts its function by binding to sterol response elements
82 (SREs) (15-17). Gain-of-function mutations in *UPC2* have been associated with
83 resistance to antifungal treatments (18-21), while strains devoid of Upc2 are
84 hypersusceptible to azole antifungals (22). One of the genes regulated by Upc2 is Erg11,
85 which is a key enzyme in the biosynthesis of ergosterol and it is the target of the azole
86 antifungal drugs. Overexpression of Erg11 has been shown to cause tolerance to these
87 drugs.
88 In order to find components linking the GdA-induced elongated growth phenotype and
89 the block of this phenotype upon overexpression of *UPC2*, we have tested a number of

compounds that were shown to mediate morphogenesis in *C. albicans*. Ascorbic acid (vitamin C) was previously shown to play a role in morphogenesis as it blocks serum-initiated hyphae formation, a process mediated by adenylate cyclase (23, 24). How ascorbic acid affects morphogenesis is not clear and we propose that it may function by modulating the role of Hsp90 in cell shape formation. In this paper, we describe a previously unreported negative effect of ascorbic acid on the Hsp90-dependent cell shape. Focusing on the mode of action of ascorbic acid, we provide evidence that it requires Upc2 and Erg11 to exert its function. We further demonstrate that intracellular ergosterol levels play a role in the ascorbic acid mediated effect on cell shape. In addition, we show that Upc2 is also required for the antagonistic effect of ascorbic acid on fluconazole toxicity. Together, these results show that ascorbic acid inhibits the Hsp90-mediated cell shape transition via the transcriptional regulator Upc2.

Materials and methods

Strains and growth conditions

Strains used in this study are listed in Table 1. *C. albicans* strains were grown overnight in 3 ml of YPD (1 % yeast extract, 2 % bacteriological peptone and 2 % glucose) at 30°C. Cells were subsequently diluted to OD_{600}=0.2 and cultured for indicated time points at 30°C with the indicated treatment (4 µM or 10 µM Geldanamycin; 2.5 mM ascorbic acid, L-cysteine, D-cysteine, glutathione or dithiotreitol (DTT), 0.1 µg/ml doxycycline). When ascorbic acid was added, the medium was buffered to pH 7. All chemicals were purchased from Sigma-Aldrich.

Transcription factor overexpression strains were grown in Complete supplement mixture minus methionine (CSM-Met medium : 0.073 % CSM-Met-Ura, 0.17 % YNB w/o amino acids and $(NH_4)_2SO_4$, 0.5 % $(NH_4)_2SO_4$, pH5.5) supplemented with 0.1 % uridine and the indicated treatment (10 μM GdA, 2.5 or 10 mM methionine).

Microscopy

Imaging of the cells was done by differential interference contrast microscopy using the Zeiss Axioplan 2 microscope. Images were obtained by a Zeiss Axiocam MRc5 camera using Axiovision software 3.0 (Carl Zeiss, Inc. NY).

Cell sedimentation assay

The cell-sedimentation rate was quantified using the assay described by Eboigbodin and Biggs (25) and Fu *et al*. (26). Cells were grown in YPD liquid medium with (0.1 μg/ml, for transcriptional repression) or without doxycycline and 2.5 mM of L-cysteine or ascorbic acid. OD_{600} was determined at indicated time points of the cells at the upper part of the glass tube, as sediments settle to the bottom of the glass tube. The formula $(OD_I - OD_t/OD_I) \times 100$ was utilized to calculate the percentage of sedimented cells. OD_I is the initial OD taken at time zero and OD_t is the OD taken at the indicated time point.

Upc2 overexpression strain: Transcription factor library

The *UPC2* overexpression strain used in this study is part of a transcription factor overexpression library, constructed in our laboratory on the basis of the previously developed *Candida albicans* two-hybrid system (27). Briefly, the nuclear localization

sequence (NLS) was removed from the one-hybrid plasmid pC2HB resulting in pC1H. Subsequently, genes encoding known and putative transcription factors were successfully cloned in fusion with the DNA-binding domain LexA in this plasmid, to create the pC1H-PTF library. The resulting plasmids were integrated in the two-hybrid reporter strain S2CH3 (27) between *XOG1* and *HOL1* loci on chromosome 1 after linearization at the *Not*I restriction site. Selection was performed on CSM-leu medium. The complete library consists of ~200 strains, each carrying an ectopically expressed protein, under the control of an inducible *MET3* promoter (Stynen *et al*., under preparation). The use of the inducible *MET3* promoter allows for a conditional expression, as it is repressed in the presence of methionine and cysteine (28).

Ergosterol extraction and quantification

Ergosterol extraction and quantification was performed as reported by Arthington-Skaggs *et al*. (29), with minor modifications. Briefly, cells were grown overnight in 4 ml YPD at 30 °C. Cells were diluted to OD_{600} =0.2 and grown in YPD medium supplemented with 10 µM GdA, 2.5 mM ascorbic acid or a combination of both. After the indicated time period, 220 ODs of cells at 600 nm [concentration of cells (OD/ml) x volume (ml) = 220 OD] were used in the heptane extraction. The same procedure was used for all experimental conditions. Cells were harvested by centrifugation at 3.000 rpm for 5 min and washed once with 10 ml sterile water. Three milliliters of 25 % alcoholic potassium hydroxide solution (25 g KOH and 35 ml sterile H_2O were brought to 100 ml with 100 % ethanol) was added to each pellet and vortexed for 1 minute. Cell suspensions were heated in an 85 °C water bath for 1 hour, then allowed to cool down at room temperature.

159 Sterols were extracted with a mixture of 1 ml of sterile water and 3 ml of n-heptane
160 followed by mixing for 3 min. The organic layer was transferred to a clean glass tube
161 and stored at -20°C for 24 h. Prior to the spectrophotometrical scan between 230 and 300
162 nm using a Shimadzu UV-1650PC spectrophotometer, sterol extracts were diluted
163 fivefold in 100% ethanol. A dilution of heptane and ethanol was used as the blank.
164 Ergosterol levels were subsequently calculated as a percentage of the wet weight as
165 described (29).
166
167 **Quantitative real-time PCR**
168 Overnight cultures of *C. albicans* strains were diluted to an OD_{600}=0.2 and cultures were
169 grown at 30 °C for the indicated time points in the presence and absence of either
170 doxycycline (0.1 µg/ml), ascorbic acid (2.5 mM) and GdA (10 µM) before RNA
171 extraction. RNA was DNase-treated prior to cDNA synthesis with the Promega A3500
172 reverse transcription kit. Quantitative real-time PCR was performed using the GoScript™
173 Reverse Transcription System (Promega) on a StepOne Plus real-time PCR system
174 (Applied Biosystems). Reactions were performed in triplicate, with oligonucleotides
175 CaERG11up, CaERG11down; Upc2_fw, Upc2_rv; Hsp90_fw, Hsp90_rv; TEF1a-fw and
176 TEF1a-rv as the primer pairs (Table 2). The fold regulation was determined using the
177 ΔΔCt method, using expression of *TEF1* to normalize the data.
178
179 **Checkerboard assay**
180 A checkerboard assay on 96-well plates was performed, by combining different
181 concentrations of ascorbic acid and fluconazole and the fractional inhibitory

concentration index (FICI) was determined. The assay was performed with both the wild type and the *UPC2* deletion mutant to verify whether the effect of ascorbic acid on fluconazole susceptibility is exerted via Upc2.

The concentrations of ascorbic acid ranged from 0.078 mM to 5 mM for both the wild type and the *UPC2* deletion mutant. The concentration of fluconazole ranged from 0.004 µg/ml to 2 µg/ml for the *UPC2* deletion mutant and 0.125 µg/ml to 64 µg/ml for the wild type. The FICI was calculated according to the formula $FIC_{index} = FIC_A + FIC_B = (MIC_{A\ comb}/MIC_{A\ alone}) + (MIC_{B\ comb}/MIC_{B\ alone})$, where $MIC_{A\ alone}$ and $MIC_{B\ alone}$ are the MICs of ascorbic acid and fluconazole alone and $MIC_{A\ comb}$ and $MIC_{B\ comb}$ are the MICs of ascorbic acid and fluconazole in combination. Interactions were referred to as synergistic when the FIC_{index} value was ≤ 0.5 and antagonistic when the FIC_{index} value was > 4. FIC_{index} values that were > 0.5 and ≤ 4 indicated no interaction between ascorbic acid and fluconazole (30). There was no difference in FIC indexes observed between 24 and 48 h.

Oxidative stress sensitivity assay

The wild type and the two independent *UPC2* deletion mutants of the Homann collection (33) were grown to mid log phase in liquid YPD medium. Cells were diluted to OD_{600} of 1 and ten-fold serially dilutions were spotted on YPD plates containing the indicated concentrations of H_2O_2. Plates were photographed after 24 hours incubation at 37°C.

Propidium iodide staining

Viability staining was performed by incubating a subset of cells (taken at the indicated time points of the experiments) for 20 min in the dark with the fluorescent stain

propidium iodide (PI; 46 mM) (Sigma-Aldrich). Prior to PI fluorescence analysis (excitation/emission maxima: 535/617 nm), cells were washed and resuspended in PBS.

Statistical analysis

Statistical significance was calculated using a student t-test analysis with ($P<0.05$ = *) and ($P<0.001$ = **). All experiments were performed with at least three biological repeats.

Results

Ascorbic acid interferes with the morphogenetic process governed by Hsp90

The transition from *C. albicans* yeast to hyphae can be induced or repressed by a wide variety of triggers (31). Partial inhibition of Hsp90, obtained by either addition of GdA, or by decreased transcription of the corresponding *HSP90* gene, results in elongated cells, which are considered neither hyphae nor pseudohyphae (13). In order to understand the mechanism by which Hsp90 affects morphogenesis, we have tested a number of compounds known to inhibit filamentation for interaction with GdA, and determined the effect of ascorbic acid on the GdA-induced elongated growth. Addition of ascorbic acid, which was previously reported to interfere with the yeast-to-hyphae transition (24), resulted in a strong reduction of the GdA-induced elongated cell shape (Figure 1A). Similar results were obtained using Radicicol, an Hsp90 inhibitor structurally distinct from GdA (32) (data not shown). Since Hsp90 is an essential chaperone, cell viability was assessed by propidium iodide (PI), a dye that is excluded from viable cells. As shown

228 in Figure 1B, the viability of GdA-treated cells is similar to that of untreated cells, ruling
229 out possible effects due to elevated cell mortality.
230 To test whether the observed effect of ascorbic acid was dependent on Hsp90, or the
231 result of chemical-chemical interference with GdA, similar experiments were conducted
232 using a strain whose Hsp90 content can be modulated via the use of a tetracycline-
233 repressible promoter (13). As expected, lowering the expression of *HSP90* with
234 doxycycline in this strain resulted in elongated growth (Figure 2A). Cell viability was
235 also tested here and indicated that cells were alive (Figure 2B). Addition of ascorbic acid
236 to cells genetically depleted of Hsp90 greatly increased the percentage of yeast cells (75
237 %), as compared to the control condition without the addition of ascorbic acid (32 %)
238 (Figure 2C). The effect of ascorbic acid was also determined using a sedimentation assay.
239 Addition of ascorbic acid to cells in a liquid culture resulted in a slower sedimentation
240 rate, indicating a higher proportion of yeast cells in these cultures as compared to
241 ascorbic acid-untreated cells (Figure 2D). Since transcription of *HSP90* in the *tetO-*
242 *HSP90* strain is reduced in the presence of doxycycline, the possibility of a chemical
243 doxycycline-ascorbic acid interaction was ruled out in experiments with wild type control
244 cells. Here, the concentration of doxycycline used in our experiments was demonstrated
245 to not interfere with the effect of ascorbic acid on the GdA-dependent elongated cell
246 shape described above (Supplementary Figure 1A). We also confirmed that doxycycline-
247 mediated repression of Hsp90 was not significantly altered in the presence of ascorbic
248 acid (Supplementary Figure 1B). Taken together, these results indicate that ascorbic acid
249 has a negative effect on elongated cell shape formation resulting from lower Hsp90
250 activity or expression.

Upc2 is required for the ascorbic acid-mediated effect.

A transcription factor deletion collection was previously used to characterize the regulatory system mediating Hsp90-regulated elongated growth (14). In a complementary approach, we have screened a transcription factor overexpression library to identify TFs that upon overexpression prevent GdA-induced morphogenesis. The library consists of ~200 strains, each carrying an ectopically expressed TF, under the control of the inducible *MET3* promoter (Stynen *et al*., under preparation). Methionine only was used to repress transcription, as cysteine was shown to interfere with elongation even in a wild type strain due to its antioxidant properties (see discussion and Supplementary Figure 2). Several transcriptional regulators whose overexpression resulted in blocked or decreased elongated growth in the presence of GdA were identified (Table 3). We continued with the strain overexpressing *UPC2* (Figure 3A), as it showed the strongest effect, based on semi-quantitative observations. Expression of *UPC2* in inducing conditions was ~4.5-fold higher than an isogenic control strain carrying an empty plasmid (Figure 3A). As expected, the characteristic elongated growth form was visible when the *MET3* promoter was repressed by the addition of methionine, confirming that increased dosage of Upc2 is responsible for the loss of elongation in the presence of inactive Hsp90 (Figure 3B).

To confirm the role of Upc2 in the Hsp90-mediated inhibition of elongated growth forms, we tested two independent strains in which the endogenous *UPC2* genes were deleted (22, 33), as such strains are expected to be hypersusceptible to GdA. Treatment of the wild type strain with low concentrations of GdA (4μM) has no effect on morphogenesis of wild type cells. However, the same concentration was sufficient to induce elongated

274 growth in the *upc2*Δ/*upc2*Δ strains, confirming their hypersusceptibility (strain D-6 is
275 shown in Figure 4A). In the complemented strain, elongation was impaired (Figure 4A).
276 Taken together, these observations indicate that Upc2 plays a role in the GdA-associated
277 elongation process, and confirm the previously observed genetic interaction between
278 Upc2 and Hsp90 (34).
279 The obvious question was then whether Upc2 is required for the effect that ascorbic acid
280 has on the Hsp90-mediated cell elongation. Addition of ascorbic acid to wild type cells
281 prevents the GdA-induced cell elongation. In the absence of Upc2 (in both independent
282 deletion strains D-6 and TF077) the GdA-induced cell elongation was not affected
283 whereas the *UPC2*-reconstituted strain EC-7 resulted in a similar phenotype as the wild
284 type strain (Figure 4B). These results indicate that the effect of ascorbic acid on the
285 morphological response upon inhibition of Hsp90 is Upc2-dependent.
286
287 **Hsp90 inhibition or depletion results in reduced intracellular ergosterol levels**
288 To determine the mechanism by which ascorbic acid and Upc2 affect elongated cell
289 growth, we tested the role of genes whose expression are regulated by Upc2, such as the
290 drug efflux pumps *CDR1* and *MDR1* (17, 35). First, we hypothesized that ascorbic acid
291 could increase the efflux of GdA, which was reported to be a substrate of the human ABC
292 (ATP-binding cassette) transporter efflux pump, Mdr1 (36, 37). If this hypothesis were
293 valid in *C. albicans*, one would expect mutants lacking Cdr1, a fungal ABC multidrug
294 transporter (38) to elongate in the presence of both GdA and ascorbic acid. In addition,
295 one would expect a strain devoid of this efflux pump to be hypersusceptible to GdA.

296 However, neither hypersusceptibility to GdA nor impairment of the morphogenetic effect
297 of ascorbic acid was observed for the *cdr1*Δ*/cdr1*Δ mutant (data not shown).
298 We also investigated Mdr1, a member of the multidrug resistance (MDR1) major
299 facilitator family. Strains overexpressing *MDR1* (39) or deleted for *MDR1* were not
300 affected in the ascorbic acid-mediated effect and were not hypersusceptible to GdA (data
301 not shown). Together, these results rule out the hypothesis that an ascorbic acid-mediated
302 increased efflux of GdA is causative for the ascorbic acid-mediated phenotype.
303 Apart from *CDR1* and *MDR1*, expression of *ERG11*, a key gene in the ergosterol
304 biosynthesis pathway, is also under the control of Upc2 (15, 22, 40). We therefore
305 reasoned that levels of Erg11 might play an instrumental role in the morphogenetic
306 transition regulated by Hsp90. The role of Erg11 on the ascorbic acid induced inhibition
307 was investigated using an engineered strain in which the only *ERG11* allele is under the
308 control of a tetracycline-repressible promoter (41). Cells, in which the transcription of
309 *ERG11* was repressed by the addition of doxycyclin, but that were still viable
310 (Supplementary Figure 3), were tested for GdA-induced elongation in the presence of
311 ascorbic acid. As shown in Figure 5A (compare a, c, g and h), Erg11 expression is
312 required for the ascorbic acid induced inhibition of cell elongation upon GdA treatment.
313 We also tested an *ERG11* overexpression strain (~3.5-fold higher expression compared to
314 the control strain) (42) but, as shown in Figure 5B, such increased dosage of *ERG11* was
315 not able to suppress the elongated phenotype upon Hsp90 inhibition, demonstrating that
316 an increased dosage of Erg11 is not sufficient to block GdA-induced elongation. We also
317 investigated whether ascorbic acid induces *ERG11* expression in the presence and
318 absence of Hsp90 inhibitors. In the wild type strain, there is a significant drop in *ERG11*

319 expression upon GdA treatment, while simultaneous addition of ascorbic acid upon this
320 treatment leads to an increased transcription of *ERG11* (Figure 5C). This increase is
321 absent in an *upc2*Δ*/upc2*Δ mutant, suggesting a role for Upc2 as an important regulator.
322 Erg11's eminent role in the regulation of sterol biosynthesis (15, 22, 43) and ascorbic
323 acid-mediated effects on the Hsp90 morphogenetic circuitry (Figure 1A and 2A),
324 suggests a possible relationship between ergosterol content and ascorbic acid induced
325 inhibition of cell elongation in the presence of GdA. To verify the impact of an impaired
326 Hsp90 function on ergosterol levels, we determined ergosterol levels in wild type and
327 *upc2*Δ*/upc2*Δ strains upon treatment with GdA (10 μM) and/or ascorbic acid (2.5 mM).
328 As shown in Figure 6A, a significant decrease in ergosterol levels can be observed after
329 addition of GdA to the wild type strain ($P < 0.05$) and the *upc2*Δ*/upc2*Δ mutant ($P <$
330 0.001) compared to the corresponding untreated control strains. Whereas ergosterol levels
331 are restored when ascorbic acid (2.5 mM) is added together with GdA to the wild type
332 strain, addition of both compounds to *upc2*Δ*/upc2*Δ cells failed to show a similar
333 restoration. The difference in ergosterol levels compared to the untreated *upc2*Δ*/upc2*Δ
334 mutant remained significant ($P < 0.05$). As can be seen, there is also a detectable
335 decrease in ergosterol levels when ascorbic acid alone is administered, but this reduction
336 is not significant ($P > 0.05$). These results point to a correlation between ergosterol levels
337 and Hsp90-governed elongated growth. To rule that reduced ergosterol levels were the
338 result of oxidative stress caused by the absence of Upc2 as was previously claimed (44),
339 we tested the two independent *upc2* mutant strains from the Homann collection (33) that
340 we used throughout our paper, for their susceptibility towards oxidative stress.
341 Supplementary figure 4 shows that the tolerance to H_2O_2 of the mutants is absolutely

342 comparable to that of the corresponding isogenic wild type strain, thus ruling out the
343 hypothesis that deletion of *UPC2* could lower the anti oxidative stress potential of the
344 cells. Different response of other *upc2* mutants to oxidative stress may have different
345 causes, such as a different genetic background (44).
346 Results obtained with pharmacological inhibition of Hsp90 were confirmed using genetic
347 depletion of Hsp90. The *tetO-HSP90/hsp90*Δ strain was grown in the presence and
348 absence of doxycycline and ergosterol levels were determined. As shown in Figure 6B,
349 depletion of Hsp90 results in a significant decrease in ergosterol levels ($P < 0.001$).
350 Similar to the pharmacological inhibition, addition of ascorbic acid results in restoration
351 of ergosterol levels, with higher ergosterol in the presence of higher ascorbic acid. To
352 rule out a more indirect effect of ascorbic acid on ergosterol levels via its antioxidant
353 effect we have analyzed ROS production by way of rhodamine fluorescence in the wild
354 type strain and in the *Tet0-HSP90/hsp90*Δ strain in the presence or absence of
355 doxycycline, and with or without ascorbic acid. Fluorescence of doxycycline-, as well as
356 of doxycycline and ascorbic acid-treated cells was comparable to that of untreated cells.
357 H_2O_2-treated cells were used as the positive control (data not shown).
358 These results suggest that decreased ergosterol levels caused by Hsp90 inhibition (Figure
359 6A and 6B) promote elongated growth. However, Figure 5A (panel d) shows that a
360 reduction of ergosterol levels (by downregulating *ERG11* expression) was not sufficient
361 to induce elongation, since elongated growth forms still required Hsp90 inhibition.
362 To our knowledge, this is the first time that a clear correlation between
363 impaired/decreased Hsp90 expression, and ergosterol levels has been observed.

364 Moreover, addition of ascorbic acid restores ergosterol levels to wild type levels and this
365 is clearer upon pharmacological inhibition, compared to genetic depletion of Hsp90.
366
367 **Upc2 is required for other ascorbic acid-mediated effects**
368 It has been reported in previous studies that ascorbic acid reduces the antifungal effect of
369 fluconazole, possibly via its antioxidant properties (45). The authors suggested that the
370 antioxidant properties of ascorbic acid counteract fluconazole-induced reactive oxygen
371 species, resulting in a similar effect obtained with other antioxidants such as glutathione
372 (45). In order to establish a general role for Upc2 in orchestrating ascorbic acid-mediated
373 phenomena, we verified a possible relationship between ascorbic acid and fluconazole in
374 a *upc2*Δ*/upc2*Δ strain by means of checkerboard assays, two-dimensional tests designed
375 to measure drug-drug interactions. Fluconazole and ascorbic acid display antagonistic
376 activity, as determined via the calculation of the fractional inhibitory concentration (FIC)
377 index (FIC index 33, Table 4). This kind of interaction is no longer detectable when the
378 checkerboard is performed using cells of the *upc2*Δ*/upc2*Δ strain (FIC index 0.75, Table
379 4). Thus, the combination of ascorbic acid with fluconazole no longer displayed
380 antagonistic activity in cells lacking the Upc2 transcription factor.
381
382 **Discussion**
383
384 Decreased dosage of Hsp90, as well as its pharmacologic depletion, results in a
385 morphology change of *C. albicans* at low temperature from yeast to elongated cells (13).
386 The molecular mechanism of this morphogenetic transition has been studied extensively

387 over the last few years (46, 47). We contribute to this characterisation by showing that
388 reduced levels of ergosterol result from GdA-mediated impairment of Hsp90, and that
389 these changes correlate with elongated growth. The effect of GdA can be suppressed by
390 addition of ascorbic acid, as this again increased levels of ergosterol to normal and
391 resulted in cell growth in the yeast form. Ascorbic acid suppression did not depend on its
392 anti oxidant activity, but depends on the transcriptional regulator Upc2, as in a
393 *upc2*Δ*/upc2*Δ mutant ascorbic acid cannot restore ergosterol levels to normal upon GdA
394 treatment. In summary, ascorbic acid can block the GdA-dependent elongated growth by
395 restoring normal ergosterol levels in a Upc2-dependent fashion.
396 In the course of our work we found that several antioxidants interfered with the activity
397 of GdA. Apart from its known inhibition of Hsp90, GdA is also involved in the
398 generation of superoxide radicals, which is attributed to the presence of its quinone group
399 (48). The presence of molecules with antioxidant properties alleviates the detrimental
400 effects of oxidative stress imposed by free radicals. A variety of molecules such as
401 reduced glutathione (GSH) and its derivatives, cysteine, dithiothreitol and ascorbic acid
402 are able to fulfil these protective requirements (49). It could be possible that the
403 superoxide radicals produced in the presence of GdA could affect the elongated cell
404 shape formation and addition of ascorbic acid would then block this through its anti
405 oxidant activities. This would fit with data obtained by Patterson et al. (50) who showed
406 that H_2O_2-mediated activation of Cap1 was a prerequisite for yeast to hyphae transition.
407 However, the fact that the antagonistic effect of ascorbic acid on morphology is observed
408 on cells genetically depleted of Hsp90 as well as on cells whose Hsp90 is inhibited by
409 GdA, suggests that ascorbic acid acts directly or indirectly on Hsp90 or Hsp90-mediated

processes. Other authors have reported an influence of ascorbic acid or its analogues on morphogenetic transitions in *C. albicans* (23, 24, 51). Nasution and colleagues (23) reported that addition of ascorbic acid not only lowered the intracellular concentration of reactive oxygen species, but also inhibited H_2O_2 and serum-initiated hyphal differentiation. Ojha and co-workers (24) similarly demonstrated inhibition of serum-induced hyphal formation by ascorbic acid and suggested a mode of action by interruption of the hyphal formation signal of *C. albicans*.

During the TF overexpression screening, we observed that cysteine directly affected the activity of GdA as no elongated growth could be observed in the wild type strain. This could be explained by earlier reports describing that thiol-containing antioxidants (such as cysteine) could physically interact with Hsp90 inhibitors, such as GdA and Radicicol, and render them inactive as a consequence of the thiol-mediated interaction (52-57). This means that one has to be careful when using the *MET3*-promoter in combination with GdA or Radicicol as in this case cysteine, that is used to repress the promoter, will affect the activity of GdA and Radicicol. This is the reason why in our experiments we used only methionine to repress the *MET3* promoter.

One of the TFs that inhibited the GdA-induced morphogenesis was Upc2, a Zn_2Cys_6 transcription factor, a key regulator of drug efflux pump expression and ergosterol biosynthesis (15, 35). Here we show that Upc2 is required for the ascorbic acid inhibition of cell elongation upon compromised Hsp90 function. Previously it was already shown that Upc2 is a key regulator of the ascorbic acid-mediated effect on fluconazole, as it decreases the antifungal effect of fluconazole, so displaying a protective role in fungi

433 (45). Together, these data show that Upc2 plays a central role as the transcriptional
434 regulator in ascorbic acid-associated phenomena. Ascorbic acid is very important for
435 human life because of its antioxidant properties, protecting cells from oxidative stress
436 (58). The use of ascorbic acid is even clinically relevant for the treatment of a variety of
437 diseases, such as respiratory tract infections (59, 60).
438 One of the genes that is regulated by Upc2 is *ERG11,* a gene involved in ergosterol
439 biosynthesis and the molecular target of the azole antifungals (15, 22, 43, 61, 62).
440 Previous work demonstrated that lower ergosterol levels, caused by ergosterol
441 biosynthesis inhibitors such as azoles, have an inhibitory effect on hyphae formation (63,
442 64). However, elongated growth forms resulting from impaired Hsp90 function are not
443 considered true hyphae (14, 65, 66). Consistent with the relationship between ergosterol
444 depletion and *C. albicans* morphogenesis mentioned by Victoria *et al*. (67), we report a
445 correlation between ergosterol content and elongated growth caused by impairment of
446 Hsp90 function. The morphogenetic switch to elongated growth forms by either
447 pharmacological inhibition or genetic depletion is accompanied with a significant
448 decrease in ergosterol content. One could question whether this drop in ergosterol is
449 really the cause or rather a consequence of the morphogenetic transition upon Hsp90
450 inhibition. The formation of elongated growth structures upon low ergosterol levels could
451 be interpreted as a cellular stress response caused by the disruption of the sterol
452 homeostasis. However, this seems unlikely, since our results argue against an elongation-
453 inducing role for low ergosterol levels. First, we demonstrated that reduced levels of
454 Erg11 are not sufficient to promote elongation (Figure 5A). In addition, it was found that
455 increasing concentrations of ascorbic acid reduced ergosterol content without initiating

456 elongated growth (Figure 6A and B). These latter data are consistent with previous
457 observations by Singh *et al*. (68), who reported low ergosterol levels in *C. albicans* cells
458 when they were grown in the presence of ascorbic acid.
459 On the other hand, the increase of ergosterol to wild type levels in the presence of GdA
460 upon addition of ascorbic acid is absent in a *upc2*Δ*/upc2*Δ mutant suggesting the
461 importance of Upc2 as a transcriptional regulator of Erg11 to overcome the drop in
462 ergosterol levels caused by impaired Hsp90 (Figure 6A). As already mentioned, we show
463 that an increased dosage of *ERG11* alone was not sufficient to overcome the induction of
464 elongated growth upon pharmacological inhibition of Hsp90. This could indicate that
465 ergosterol biosynthesis is not the most important Upc2-mediated effect in response to
466 ascorbic acid or that the expression other *ERG* genes could be regulated by Upc2. We are
467 currently identifying other Upc2-regulated genes by performing ChIP-Seq and RNA-seq
468 analysis. We cannot exclude a possible connection with the previously established
469 pathways in the Hsp90-dependent morphogenetic circuitry, such as the cAMP-PKA and
470 cell cycle pathways or the Pho85-Pcl1-Hms1 module (13, 14, 46, 65, 69) or a novel, yet-
471 to-be-determined pathway. Ascorbic acid or Upc2 may be required for the modulation of
472 an inhibitor of these Hsp90 signalling modules.
473
474 In conclusion, our study elucidates the molecular circuitry through which ascorbic acid
475 influences the Hsp90-dependent *C. albicans* morphogenesis, involving the transcriptional
476 regulator Upc2. We find that ascorbic acid can block the GdA-dependent elongated
477 growth by restoring normal ergosterol levels in a Upc2-dependent fashion. We suggest
478 that influencing ergosterol biosynthesis via Erg11 is not the primary site of action of

Upc2-mediated effects in response to ascorbic acid, as overexpression of *ERG11* did not block elongation upon GdA-treatment. Further research is required to further explore the interesting relationship between Hsp90, Upc2, Erg11 and the morphogenetic machinery.

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718 **Figure legends**

719

720 **Figure 1: Ascorbic acid abrogates GdA-induced elongated growth**

721 A) Inhibiting effect of ascorbic acid on elongated growth induced by 10 μM GdA. Wild
722 type SC5314 cells were grown at 30 °C in liquid rich medium containing GdA (10 μM)
723 with 2.5 mM ascorbic acid. Pictures were taken after 8 h of growth. B) GdA-mediated
724 pharmacological inhibition of Hsp90 for extended periods of times is not lethal.
725 Propidium Iodide stained heat-killed wild type cells (15 min at 80 °C) are clearly
726 illustrating a complete lethal phenotype as indicated by the staining (which is excluded
727 from viable cells), while the GdA-treated cells closely resemble the untreated wild type
728 cells. Pictures were taken after 24 h.

729

730 **Figure 2: Ascorbic acid influences phenotypes caused by Hsp90 genetic depletion**

731 A) Ascorbic acid (2.5 mM) counteracts elongated growth upon genetic depletion of
732 Hsp90 (0.1 μg/ml Dox). Pictures were taken after 24 h. B) Genetic depletion of Hsp90 for
733 extended of times in the periods of times in the presence of 0.1 μg/ml doxycycline is not
734 completely lethal. Propidium iodide stained heat killed wild type cells (15 min at 80 °C)
735 are clearly illustrating a complete lethal phenotype as indicated by the staining, while the
736 cells genetically depleted of Hsp90 closely resemble the untreated wild type cells.
737 Pictures were taken at 24 h. C) The percentage of yeast cells grown for 24 h in the
738 presence of ascorbic acid was significantly (**$p < 0.001$) higher compared to the
739 untreated condition D) The sedimentation rate was monitored of cultures of a *Candida*
740 strain in which the remaining *Hsp90* was under control of a tetracycline-repressible

promoter in liquid YPD rich medium supplemented with 0.1 µg/ml Dox (■), 0.1 µg/ml Dox + 2.5 mM L-Cysteine (◇), 0.1 µg/ml Dox + 2.5 mM ascorbic acid (▲) and 0.1 µg/ml Dox + 2.5 mM Glutathione (●). The graphs were plotted by percentage of cells sedimented against time.

Figure 3: Overexpression of *UPC2* represses GdA-induced elongated growth

A) Quantitative real-time PCR of *UPC2* expression under the control of the inducible *MET3* promoter, in the *UPC2* overexpression strain and the control strain carrying an identical construct with the exception of the transcription factor. Promoter repressing conditions in the presence of 10 mM methionine are represented in white, while the black bars represent inducing conditions. The graph shows mean values with standard deviations from two independent experiments. B) The inducible *MET3* promoter allows regulated expression. Overexpression of *UPC2* in the presence of GdA (10 µM) blocks the rise of the characteristic elongated growth form. This yeast form is reversed to the elongated structures under promoter repressing conditions (represented by addition of 10 mM methionine). The control strain carrying an identical expression construct except the transcriptional regulator, displays a filamentous growth form in both promoter repressing and inducing conditions. Pictures were taken after 8 h of growth at 30 °C in CSM-met medium.

Figure 4: The *UPC2* deletion strain is hypersusceptible for GdA and impairs ascorbic acid associated blockage of the elongated cell shape

763 A. The *UPC2* deletion strain is hypersusceptible to GdA. The *upc2*Δ*/upc2*Δ (D-6) strain
764 was tested for its hypersusceptibility to GdA by treating them with 4 μM of GdA, a
765 concentration that is unable to induce elongated growth in the corresponding wild type.
766 The *UPC2* re-integrant strain (EC-7) displays a similar phenotype as the wild type.
767 Pictures were taken after 8 h of growth at 30 °C in YPD medium. B. Deletion of *UPC2*
768 impairs ascorbic acid associated blockage of the elongated cell shape. Deletion strains
769 were grown at 30 °C in YPD rich medium supplemented with either 10 μM GdA, 2.5
770 mM L-ascorbic acid or a combination of both. While ascorbic acid blocks the GdA-
771 induced filamentation of the wild-type strain, it is unable to do so in the *upc2*Δ*/upc2*Δ
772 mutants D-6 and TF077, while the reconstitution of *UPC2* restores the yeast growth
773 response. Pictures were taken after 8 h.
774
775 **Figure 5: *ERG11* is required for the ascorbic acid-mediated effect**
776
777 A) A *tetO-ERG11/erg11*Δ was grown at 30 °C in YPD rich medium supplemented with
778 either 10 μM GdA, 2.5 mM L-ascorbic acid, 0.1 μg/ml doxycycline or a combination of
779 these substances. While ascorbic acid blocks elongation upon GdA-treatment of the *tetO-*
780 *ERG11/erg11*Δin the absence of doxycycline, compromising *ERG11* expression is able to
781 overcome this ascorbic acid associated blockage. Pictures were taken after 8 h. B)
782 Overexpression of *ERG11* is not sufficient to block elongated growth caused by Hsp90
783 inhibition. *C. albicans* strains AFA60 (control) and AFA59b (*ACT1p-CaERG11)* were
784 grown at 30 °C in YPD rich medium supplemented with 10μM GdA. GdA-induced
785 elongation of both the control and the *ACT1p-CaERG11* strain shows that an increased

786 dosage of Erg11 cannot suppress elongation. Pictures were taken after 8 h. C) There is a
787 significant drop (*p < 0.05) in *ERG11* expression upon GdA treatment in the wild type,
788 while simultaneous addition of ascorbic acid upon this treatment leads to an increase.
789 Interestingly, this latter observation is absent in the *upc2*Δ*/upc2*Δ mutant, suggesting a
790 role for Upc2. Data are means of ± standard deviations of triplicate experiments.
791
792 **Figure 6: Hsp90 inhibition or depletion result in reduced intracellular ergosterol**
793 **levels**
794 A) Ergosterol measurements in the wild type and *upc2*Δ*/upc2*Δ mutant show a significant
795 decrease in ergosterol content after pharmacological inhibition of Hsp90 in comparison
796 with the representative untreated control (*p < 0.05; **p < 0.001). Addition of ascorbic
797 acid under these conditions was reflected by an increase of ergosterol content only in the
798 wild type. Results are means of at least three independent measurements. Legend: Black:
799 untreated; Dark grey: 2.5 mM ascorbic acid; Light grey: 10 μM GdA; White: 10 μM
800 GdA + 2.5 mM ascorbic acid. Data are means of ± standard deviations of triplicate
801 experiments.
802 B) Ergosterol measurements in the *tetO- HSP90/hsp90*Δ strain show a similar significant
803 decrease in ergosterol content in the presence of doxycycline (0.1 μg/ml) compared to the
804 untreated control (*p < 0.05; **p < 0.001). This drop in ergosterol levels has great
805 similarities with pharmacological inhibition by GdA. Addition of ascorbic acid is
806 reflected by an increase in ergosterol content in the presence of doxycycline, while there
807 is a general decrease noticeable in its absence. Data are means of ± standard deviations of
808 triplicate experiments.

809 **Tables**

810 **Table 1 : Strains used in this study**

811

Strain	Description	Genotype	Source
SC5314		Wild type	(70)
CAI4		*ura3*Δ*::imm434/ura3*Δ*::imm434 iro1*Δ*/iro1*Δ*::imm343*	(71)
AFA60a	CAI4 transformed with *URA3* vector	*ura3*Δ*::imm434/ura3*Δ*::imm434 iro1*Δ*/iro1*Δ*::imm343 RPS10::CIp10*	(42)
AFA59b	CAI4 carrying extra copy of *CaERG11* under control of *ACT1* promoter	*ura3*Δ*::imm434/ura3*Δ*::imm434 iro1*Δ*/iro1*Δ*::imm343 RPS10::pAFC89b*	(42)
DSY448	*cdr1*Δ*/cdr1*Δmutant	*ura3*Δ*::imm434/ura3*Δ*::imm434 iro1*Δ*/iro1*Δ*::imm343 cdr1*Δ*::hisG-URA3-hisG/cdr1*Δ*::hisG*	(72)
DSY465	*mdr1*Δ*/mdr1*Δmutant	*ura3*Δ*::imm434/ura3*Δ*::imm434 iro1*Δ*/iro1*Δ*::imm343 ben*Δ*::hisG-URA3-hisG/ben*Δ*::hisG*	(72)
CMDR1E2 A and -B	CAI4 carrying *CaMDR1* under control of *ADH1* promoter	*ura3*Δ*::imm434/ura3*Δ*::imm434 iro1*Δ*/iro1*Δ*::imm343 ADH1/adh1*::P_{ADH1}*-MDR1-URA3*	(39)
SN152	Background strain TF wild type and TF077	*arg4*Δ*/arg4*Δ *leu2*Δ */leu2*Δ *his1*Δ*/his1*Δ *URA3/ura3*Δ*::imm434 IRO1/iro1*Δ*::imm343*	(73)
TF wild type	Control wild type strain (paired "wild type" TF077)	*arg4*Δ */arg4*Δ *leu2*Δ */LEU2 his1*Δ*/HIS1 URA3/ura3*Δ*::imm434 IRO1/iro1*Δ*::imm343*	(33)

TF077	*upc2*Δ*/upc2*Δmutant	As SN152, but *upc2*Δ*::HIS1/upc2*Δ*::LEU2*	(33)
BWP17 (TW14901)	Background strain D-6 and EC-7	*ura3*Δ*::imm434/ura3*Δ*::imm434 iro1/iro1*Δ::imm434 *his1*Δ*::hisG/his1*Δ*::hisG arg4*Δ*/arg4*Δ	(22)
D-6 (TW14903)	*upc2*Δ*/upc2*Δmutant	As BWP17 but *upc2*Δ*::URA3/upc2*Δ*::ARG4*	(22)
EC-7 (TW14904)	Reconstituted strain	As D-6 but *upc2*Δ*::URA3/upc2*Δ*::UPC2-HIS1*	(22)
pTET-ERG11	CAI4 with doxycycline-repressible *ERG11* as the only *ERG11* allele	As CAI4, but *tetO-ERG11/erg11*Δ	(41)
CaLC1411 (CaLC436)	Strain with doxycycline-repressible *HSP90* as the only *HSP90* allele	*arg4*Δ*/arg4*Δ*his1*Δ*/his1*Δ*URA3/ura3*Δ*::imm434 IRO1/iro1*Δ::*imm434 HIS1/his1*Δ*::TAR-FRT hsp90*Δ*::CdHIS1/his1*Δ*::TAR-FRT*	(74)
SC2H3		As SN152, but 5xLexAOp-ADH1b/HIS1 5xLexAOp-ADH1b/lacZ	(27)
OX Control	Strain carrying empty vector under *MET3* promoter	As SC2H3, but *MET3pro:lexA*	Stynen *et al*., under preparation
Upc2 OX	Strain carrying extra copy of *UPC2* under *MET3* promoter	As SC2H3, but *MET3pro:lexA:UPC2*	Stynen *et al*., under preparation
Gcn4 OX	Strain carrying extra copy of	As SC2H3, but	Stynen *et*

	GCN4 under *MET3* promoter	*MET3pro:lexA:GCN4*	*al*., under preparation
Cap1 OX	Strain carrying extra copy of *CAP1* under *MET3* promoter	As SC2H3, but *MET3pro:lexA:CAP1*	Stynen *et al*., under preparation
Dot6 OX	Strain carrying extra copy of *DOT6* under *MET3* promoter	As SC2H3, but *MET3pro:lexA:DOT6*	Stynen *et al*., under preparation
Rtg3 OX	Strain carrying extra copy of *RTG3* under *MET3* promoter	As SC2H3, but *MET3pro:lexA:RTG3*	Stynen *et al*., under preparation

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815 **Table 2: Primers used in this study**

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Primer	Description	Source
CaERG11 up	TTACCTCATTATTGGAGACGTGATG	(42)
CaERG11 down	CACGTTCTCTTCTCAGTTTAATTTCTTTC	(42)
TEF1a_fw	CCACTGAAGTCAAGTCCGTTGA	(42)
TEF1a-rv	CACCTTCAGCCAATTGTTCGT	(42)
UPC2_fw	GGATGCTCGACATGCAAAAAG	This study
UPC2_rv	TGCCACATACAGGTCTCTGTTCA	This study
HSP90_fw	CCATCTGATATCACTCAAGATG	This study
HSP90_rv	AGTGATAAACACTCTACGGACG	This study

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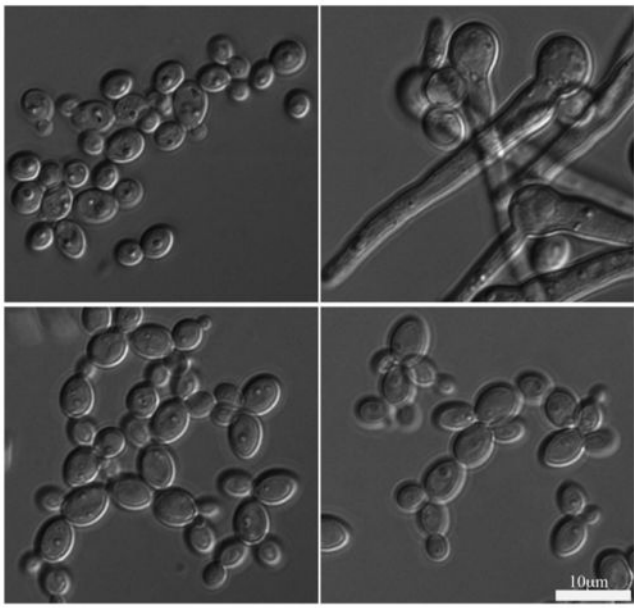
Table 3: Transcription factors that upon overexpression block the GdA-induced elongated growth.

Upc2	Zn_2-Cys_6 transcriptional regulator of ergosterol biosynthetic genes and sterol uptake
Cap1	AP-1 family bZIP transcription factor involved in drug resistance and oxidative stress regulation
Dot6	Uncharacterized ORF, protein with a predicted role in telomeric gene silencing and filamentation
Rtg3	Uncharacterized ORF, putative transcription factor with bZIP DNA-binding motif
Gcn4	bZIP transcription factor involved in amino acid control response

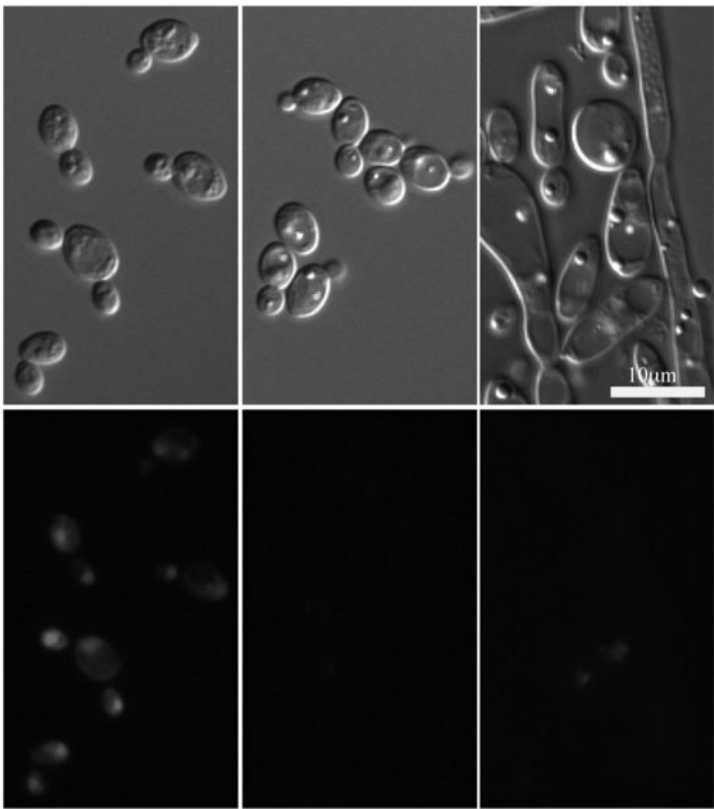
Table 4: The antagonistic effect of ascorbic acid on fluconazole is not present in a *upc2*Δ/*upc2*Δ mutant. MIC_{FLC} and MIC_{AA} are MICs of fluconazole and ascorbic alone, while $MIC_{FLC/AA}$ and $MIC_{AA/FLC}$ are MICs of ascorbic acid and fluconazole in combination. Interpretation of the FIC index was performed as previously described (30).

Strain	MIC $_{FLC}$	MIC $_{FLC/AA}$	MIC $_{AA}$	MIC $_{AA/FLC}$	FIC index
Wild type	2 µg/ml	>64 µg/ml	1761.2 µg/ml (10 mM)	1761.2 µg/ml (10 mM)	33
*upc2*Δ/*upc2*Δ	0.25 µg/ml	0.125 µg/ml	1761.2 µg/ml (10 mM)	440.3 µg/ml (2.5 mM)	0.75

A


- GdA
10 μM GdA
Untreated
2.5 mM Ascorbic acid
10μm

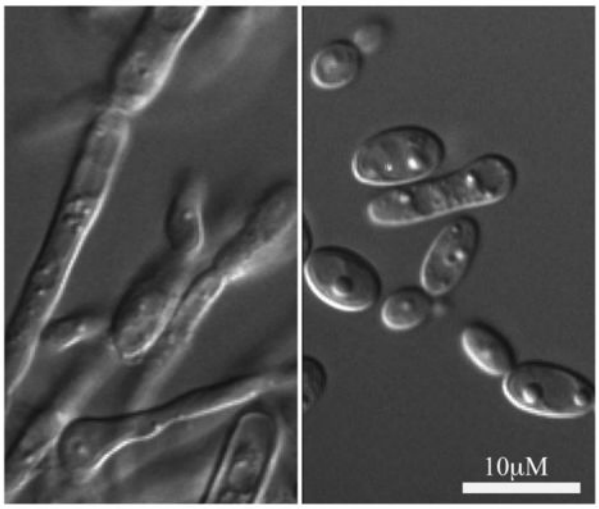
B


Heat killed
Wild type control
Wild type
Untreated
Wild type
10 μM GdA
10μm

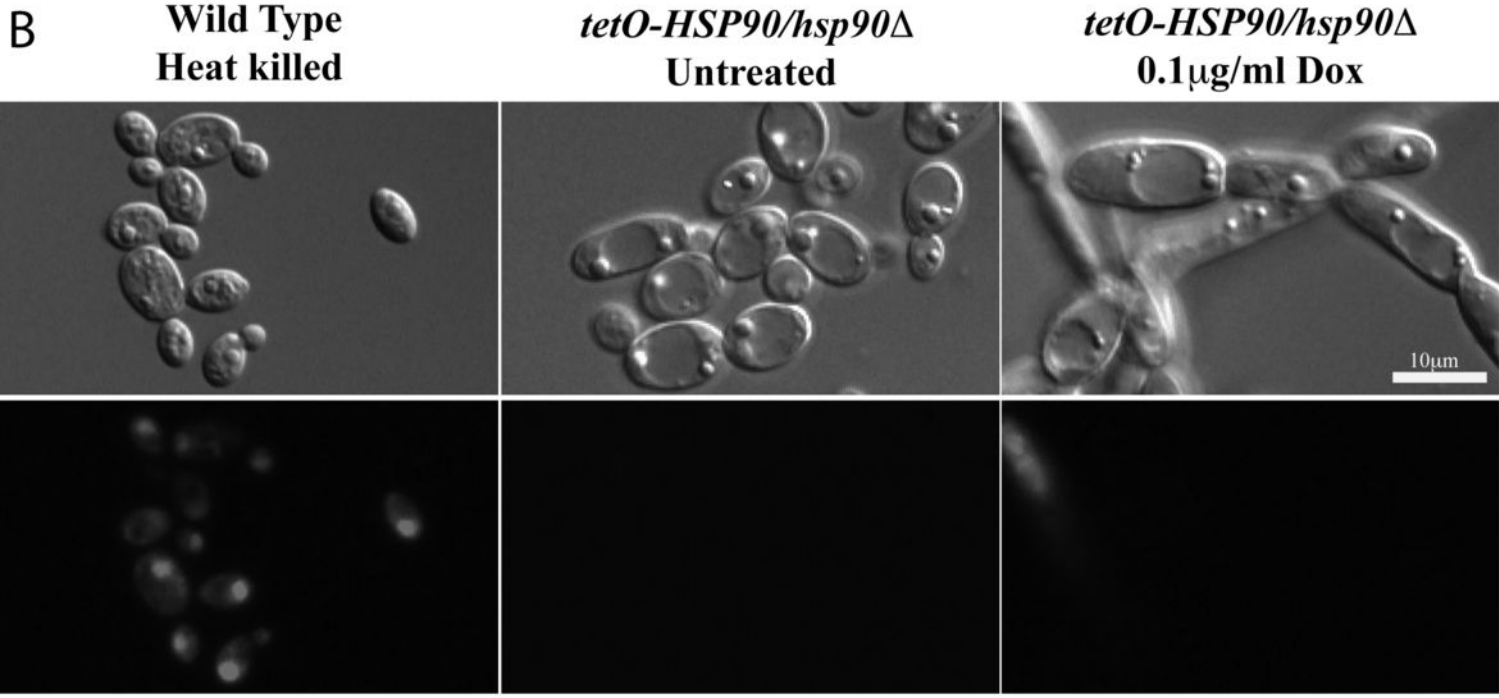
A

0.1μg/ml Dox	+	+
2.5mM Ascorbic Acid	-	+

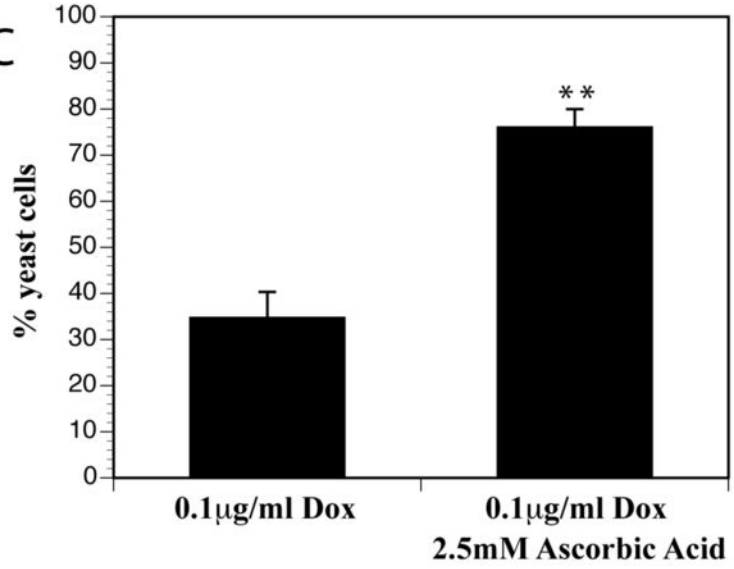
tetO-HSP90/hsp90Δ



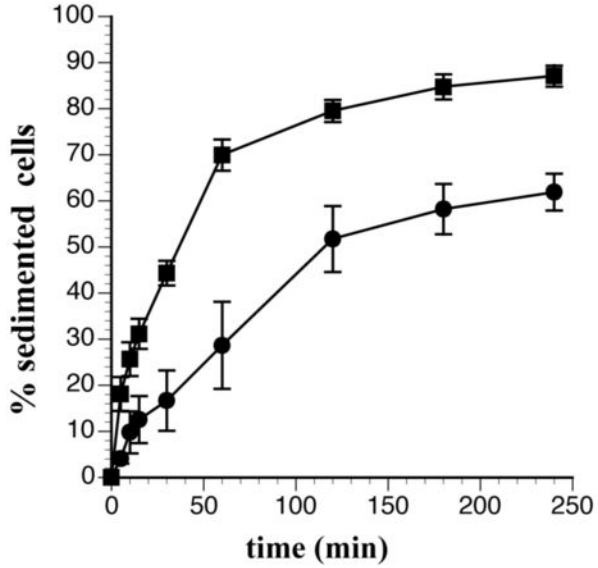
B



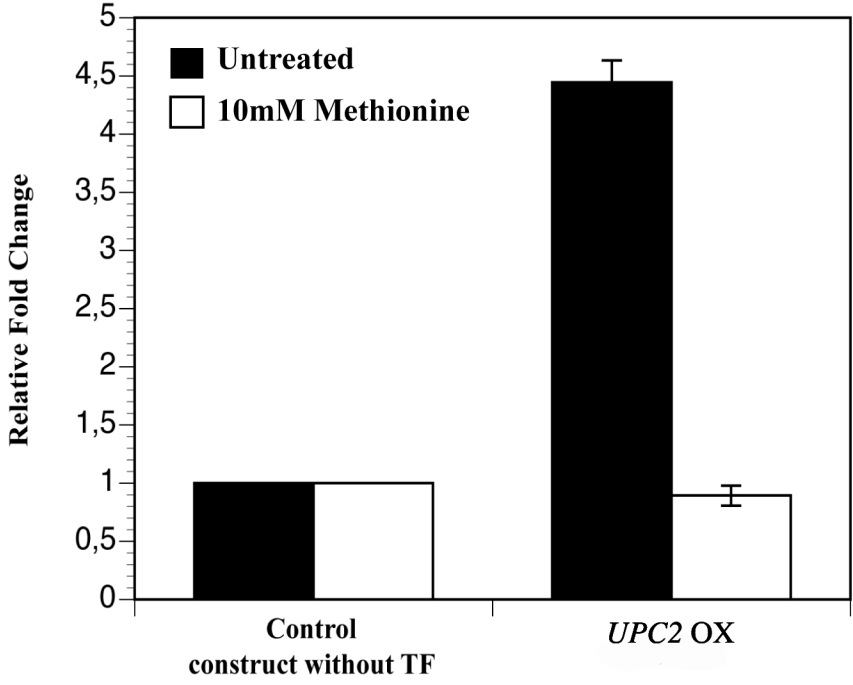
C



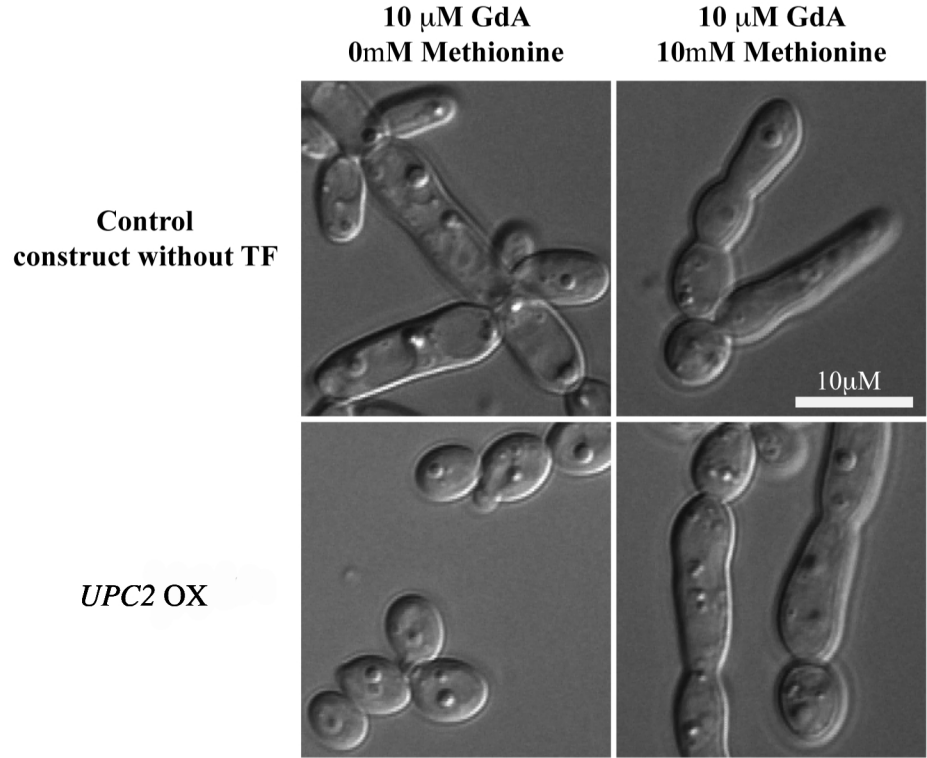
D

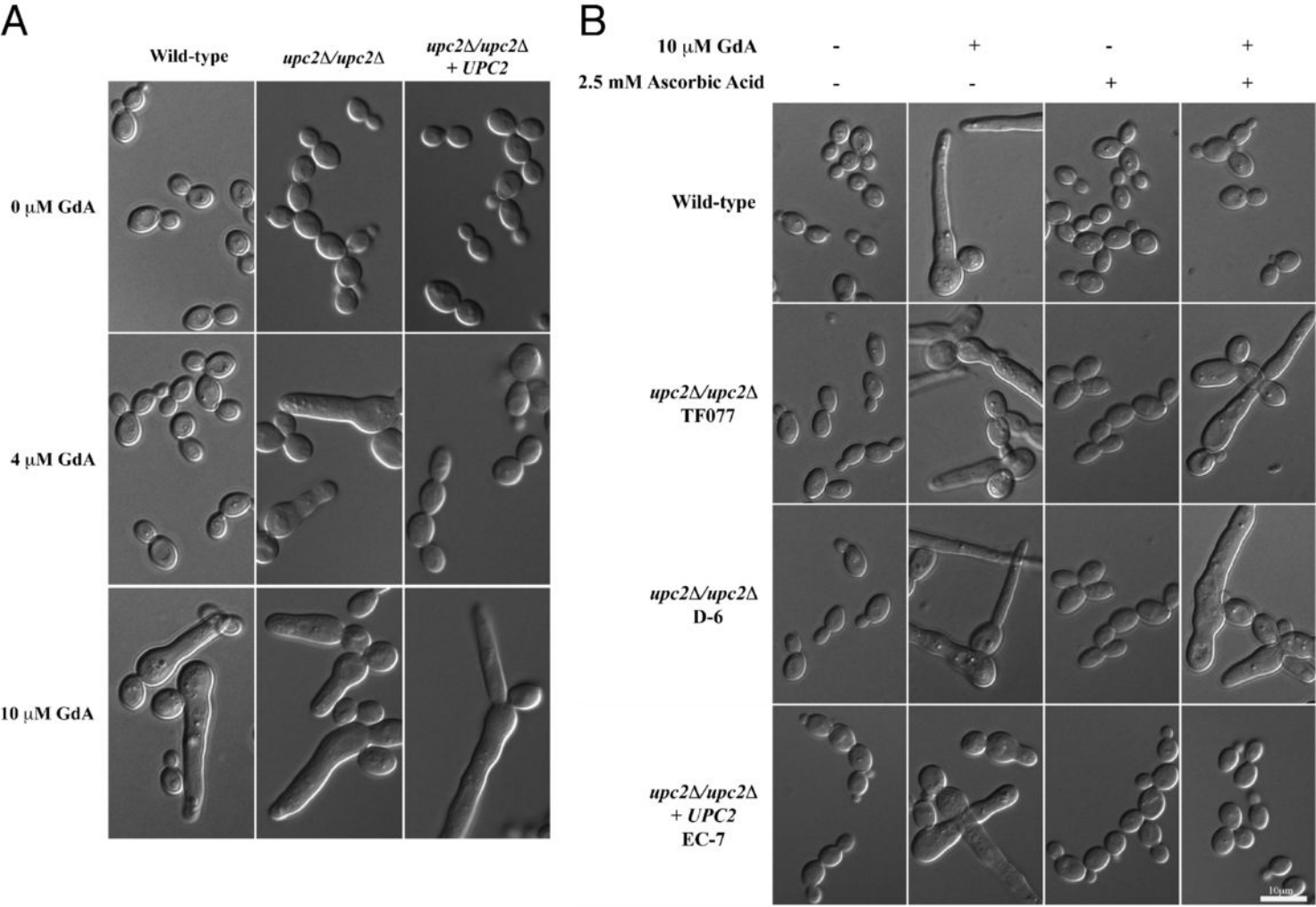


A


Untreated
10mM Methionine
Relative Fold Change
5
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
Control
construct without TF
UPC2 OX

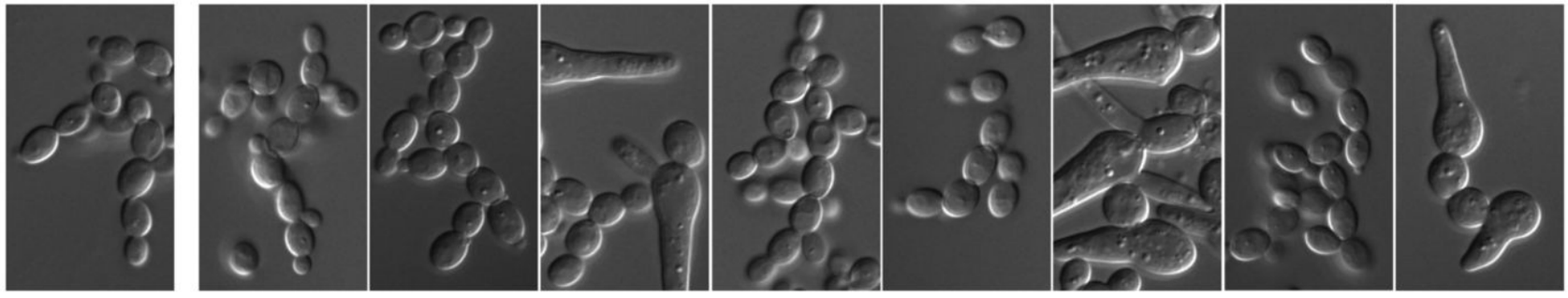
B


10 μM GdA
0mM Methionine
10 μM GdA
10mM Methionine
Control
construct without TF
10μM
UPC2 OX


A
Wild-type
upc2Δ/upc2Δ
upc2Δ/upc2Δ + UPC2
0 μM GdA
4 μM GdA
10 μM GdA
B
10 μM GdA - + - +
2.5 mM Ascorbic Acid - - + +
Wild-type
upc2Δ/upc2Δ TF077
upc2Δ/upc2Δ D-6
upc2Δ/upc2Δ + UPC2 EC-7
10μm

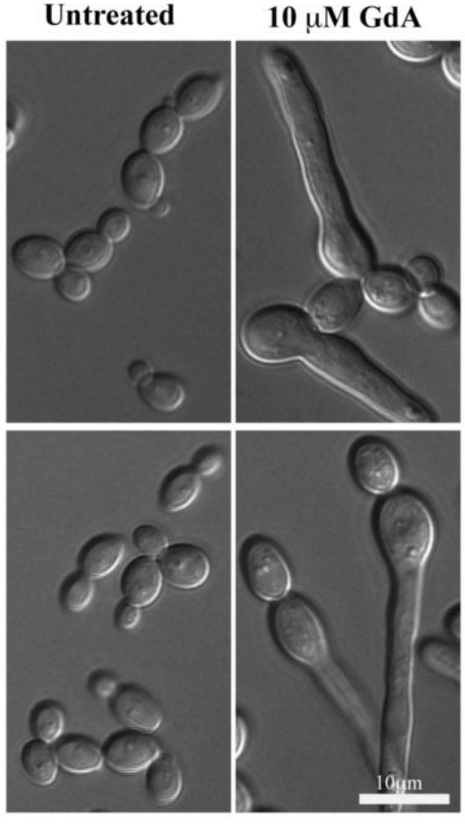
A

	wild type	*tetO-ERG11/erg11Δ*							
0.1μg/ml Dox	+	-	-	-	+	+	+	-	+
2.5mM Ascorbic Acid	-	-	+	-	-	+	-	+	+
10μM Geldanamycin	-	-	-	+	-	-	+	+	+

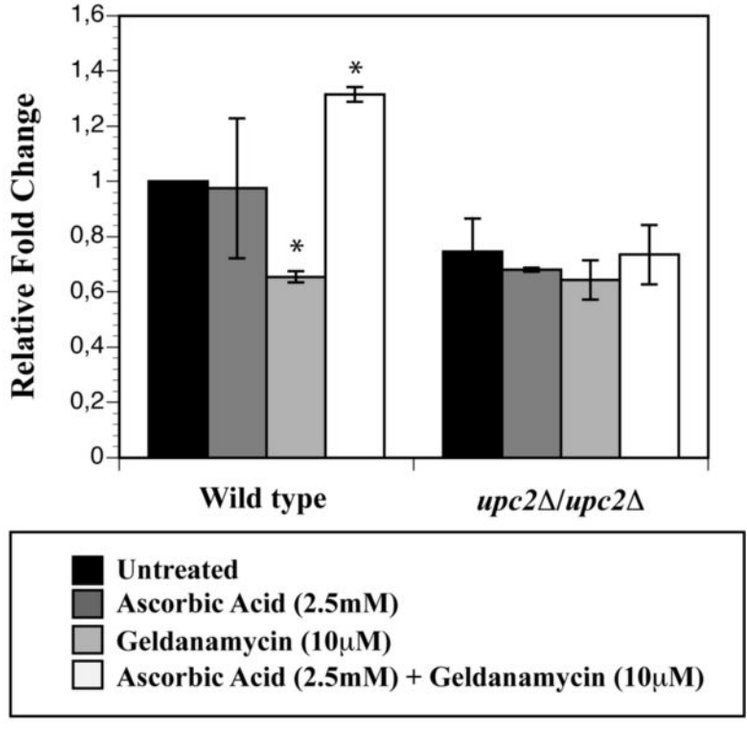


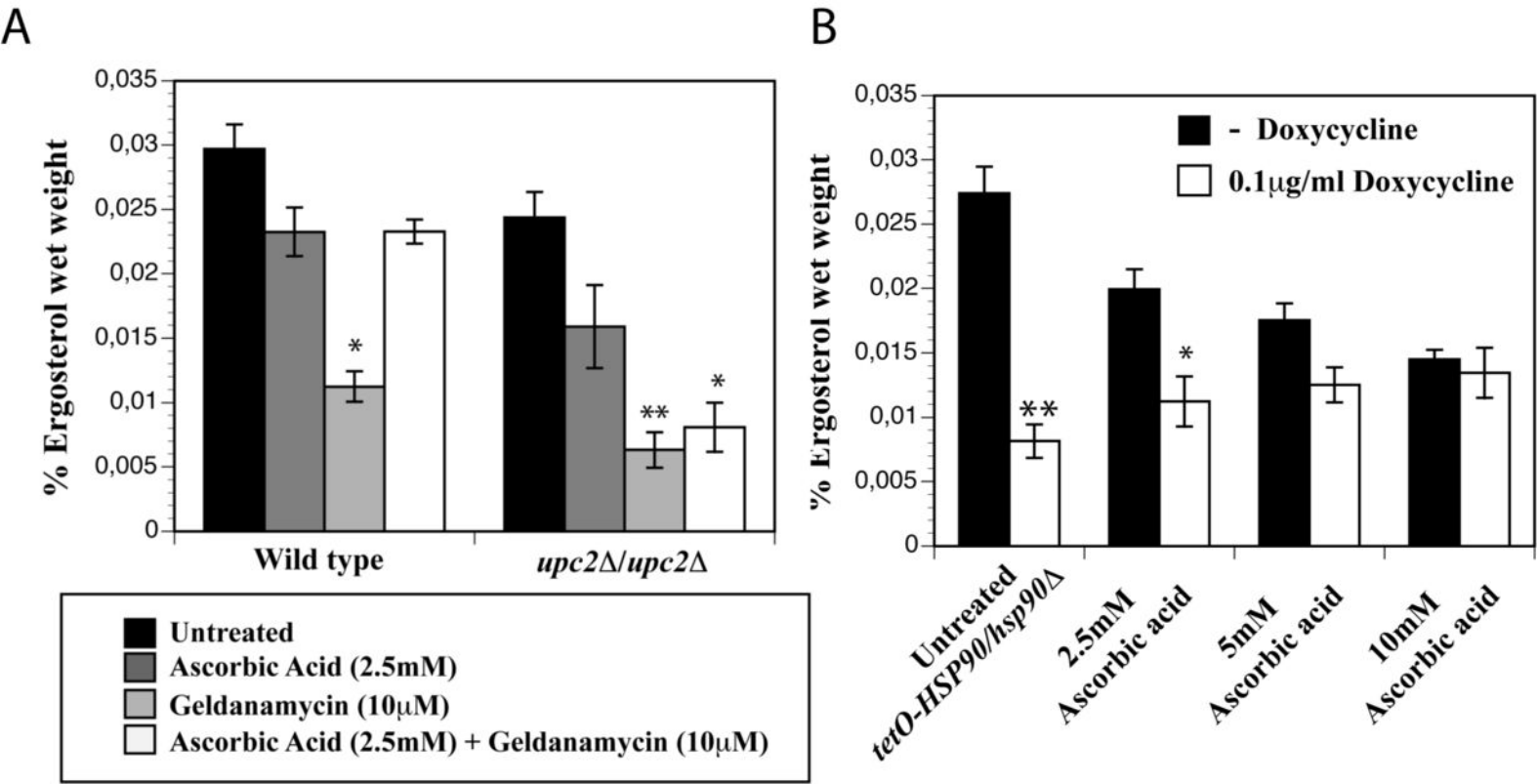
a b c d e f g h

B



C




A
0,035
0,03
0,025
0,02
0,015
0,01
0,005
0
% Ergosterol wet weight
*
**
*
Wild type
upc2Δ/upc2Δ
Untreated
Ascorbic Acid (2.5mM)
Geldanamycin (10μM)
Ascorbic Acid (2.5mM) + Geldanamycin (10μM)
B
0,035
0,03
0,025
0,02
0,015
0,01
0,005
0
% Ergosterol wet weight
- Doxycycline
0.1μg/ml Doxycycline
**
*
Untreated tetO-HSP90/hsp90Δ
2.5mM Ascorbic acid
5mM Ascorbic acid
10mM Ascorbic acid